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BROOD PRODUCTION AND LINEAGE DISCRIMINATION IN THE RED HARVESTER ANT (*POGONOMYRMEX BARBATUS*)

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Abstract. In contrast to the system of caste determination in most social insects, reproductive caste determination in some populations of *Pogonomyrmex barbatus* has a genetic basis. Populations that exhibit genetic caste determination are segregated into two distinct, genetic lineages. Same-lineage matings result in female reproductives, while inter-lineage matings result in workers. To investigate whether founding *P. barbatus* queens lay eggs of reproductive genotype, and to determine the fate of those eggs, we genotyped eggs, larvae, and pupae produced by naturally inseminated, laboratory-raised queens. We show that founding dependent lineage queens do lay eggs of reproductive genotype, and that the proportion of reproductive genotypes decreases over the course of development from eggs to larvae to pupae. Because queens must mate with a male of each lineage to produce both workers and female reproductives, it would benefit queens to be able to distinguish males of the two lineages. Here we show that *P. barbatus* males from the two genetic lineages differ in their cuticular hydrocarbon profiles. Queens could use male cuticular hydrocarbons as cues to assess the lineage of males at the mating aggregation, and possibly keep mating until they have mated with males of both lineages.

Key words: caste determination; cuticular hydrocarbons; harvester ants; Pogonomyrmex barbatus; *reproductive allocation.*

Special Feature

Introduction

Until the discovery of an association between genotype and caste in harvester ants (Helms Cahan et al. 2002, Julian et al. 2002, Volny and Gordon 2002*a*), it was generally accepted that the differentiation of female social Hymenoptera (ants, bees, and wasps) into sterile workers and reproductive queens was determined by environmental factors such as nutrition and incubation temperature during development (Wilson 1971, Michener 1974). Bi-potent eggs and environmental caste determination would allow colonies to allocate resources toward the production of workers or reproductives as needed. Indeed, prior to the discovery of a genetic system of caste determination, environmental caste determination seemed not only plausible, but necessary, because genes that result in worker sterility would not be passed on to the next generation, and would thus be eliminated from the population (Bourke and Franks 1995).

Recent work on *Pogonomyrmex* harvester ants has demonstrated that reproductive caste determination in some populations of *P. barbatus,* as well as *P. rugosus,* has a genetic basis (Helms Cahan et al. 2002, Julian et al. 2002, Volny and Gordon 2002*a*, Helms Cahan and Keller 2003). Populations in which reproductive caste determination has a genetic basis are separated into two distinct, genetic lineages. Matings between queens and males from different lineages result in sterile workers, while matings between queens and males from the same lineage result in female reproductives (virgin queens). Males, which are haploid, develop from unfertilized eggs laid by queens from either lineage. Following Anderson et al. (2006), we refer to the two genetic lineages as lineage J1 and lineage J2 (Helms Cahan and Keller 2003), which correspond to lineages X and 4 in Volny and Gordon (2002*a*), respectively.

Harvester ant colonies reproduce by sending winged reproductives (males and virgin queens) to an annual mating flight. After mating with one or more males, queens leave the mating aggregation to found new colonies. A range of two to six mates has been observed in the study population (Volny 2003). Each colony is founded by a single queen. Throughout her lifetime, the queen uses sperm from her original mating to produce all of the colony's workers and reproductives. In this system of caste determination, queens must mate at least twice, with a male from each lineage, to produce both workers and female reproductives. A queen that mates exclusively with males from the opposite lineage will be able to produce workers, but all of her reproductive offspring will be male. A queen that mates only with males from her own lineage will not be able to produce

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workers, which are needed to collect food, build a nest, and care for the brood.

The two-lineage genetic system raises interesting questions about the timing of the production of sterile workers and female reproductives. A long-term study of a two-lineage population of *P. barbatus* shows that a queen lives for 15–20 years (Gordon 1991), but the lifespan of a worker does not exceed one year (Gordon and Hölldobler 1987). Thus, a queen must continuously produce workers to reach and maintain a mature colony size of 10 000–12 000 workers (Gordon 1992). In contrast, reproductives need not be produced continuously. In the two-lineage population under study, *P. barbatus* colonies do not produce reproductives until they are about five years old (Gordon 1995). Once a colony is mature, reproductives are produced only seasonally in preparation for the annual mating flight, as in other *Pogonomyrmex* species (MacKay 1981). The two-lineage system of caste determination affects reproductive allocation, the division of resources between colony maintenance (the production of workers) and reproduction (the production of reproductives). If sperm use is random, multiply mated queens will lay same-lineage eggs destined to become reproductives at inappropriate times, for example during the colony founding stage and outside the reproductive season. Helms Cahan et al. (2004) have shown that founding queens exclusively raise workers, even when they have only mated with a single male of the same lineage. This system has parallels with diploid male production following matings between individuals carrying the same alleles at one or more sex-determining loci (Crozier and Page 1985). Such diploid males are sterile, and thus are a waste of colony resources. A selective pressure for polyandry has been proposed as a means of reducing the variance in diploid male production among colonies. Similarly, in two-lineage populations of harvester ants, a selective pressure for multiple mating would reduce the frequency of queens mated exclusively with males of the same lineage.

Pre-zygotic selection mechanisms such as sperm precedence or sperm choice by the queen could account for the absence of adult reproductives in young colonies. Incomplete mixing of sperm from different males in the queen's spermatheca could result in the exclusive production of workers if the first male to fertilize a founding queen's eggs is always of the opposite lineage. Alternatively, sperm choice could allow founding queens to control the production of workers and female reproductives. However, such pre-zygotic selection mechanisms are considered very unlikely in ants (Boomsma et al. 2005).

Post-zygotic selection is an alternative explanation for why adult reproductives are produced only in mature colonies and only during the reproductive season. The queen or workers could control reproductive allocation by eliminating reproductive eggs, or selectively feeding or neglecting certain larvae. However, without any pre-zygotic selection, the initial ratio of worker to reproductive eggs laid by the queen would depend on the genotypes and number of males with which the queen mated.

To examine whether pre- or post-zygotic selection mechanisms account for the absence of adult reproductives in founding colonies, we brought naturally inseminated, founding *P. barbatus* queens into the laboratory and genotyped their brood. Here we ask: (1) Do naturally inseminated, founding *P. barbatus* queens from a two-lineage population lay same-lineage eggs? and (2) If so, does the proportion of same-lineage eggs change over the course of development from eggs to larvae to pupae? Our approach, like that of Clark et al. (2006), consists of examining the eggs laid by founding queens. Unlike Helms Cahan et al. (2004), we did not genotype the queens. The absence of same-lineage genotypes among newly laid eggs would indicate that either queens are able to select sperm, or an extreme form of sperm precedence is in effect. The presence of reproductive genotypes, on the other hand, would indicate that sperm selection or sperm precedence do not account for the absence of reproductives at certain times in a colony's life cycle. Furthermore, a reduction in the proportion of reproductive genotypes over the course of development would indicate a post-zygotic selection mechanism.

Because of the apparent cost of same-lineage matings, the ability to distinguish males of the two lineages could be beneficial for queens (Ashe and Oldroyd 2002). If there is a proximate basis by which queens can identify male lineage, they could at least ascertain that they have mated with a male of each lineage before leaving the mating aggregation, even of they do not control mate choice. Here we ask whether males of the two lineages differ in their cuticular hydrocarbon profiles. Communication in social insects occurs predominantly by tactile and chemical means (Hölldobler and Wilson 1990). Cuticular hydrocarbons, in particular, appear to be the most important class of compounds used as recognition cues for many social insect species (Lenoir et al. 1999, Howard and Blomquist 2005). Cuticular hydrocarbons have been demonstrated to be used by social insects as recognition cues to determine species and colony membership (Bagnères et al. 1991, Lahav et al. 1999, Thomas et al. 1999, Wagner et al. 2000, Dani et al. 2001, Ruther et al. 2002). Within colonies, hydrocarbon-based recognition cues are used to recognize task membership and reproductive status (Dietemann et al. 2003, Greene and Gordon 2003). If males of the two lineages differ in their cuticular hydrocarbon profiles, queens may be able to distinguish between males from each lineage.

METHODS

Collection of males and queens

Males and mated queens were collected from a two-lineage population of *P. barbatus* colonies that has been the subject of an ongoing behavioral and demographic

study for the past 22 years (Gordon and Kulig 1996). The study population is located near Rodeo, New Mexico, USA. This population is located approximately 2 km north of site J and 4 km south of site H in Helms Cahan and Keller (2003) and Helms Cahan et al. (2004), and approximately 75 km south-southwest of Lordsburg, New Mexico, where population 10 of Anderson et al. (2006) and the collection site of Clark et al. (2006) is located. For studies of reproductive allocation, mated queens were collected on 23 and 24 July, 2002, after they had left the mating aggregation to found new colonies. Their insemination status was apparent because they had shed their wings (Hölldobler 1976). Each queen was placed in a petri dish containing a moist cotton ball, and was kept at ambient temperature before and during transport to Stanford University. Once in the laboratory, queens were kept in a constant temperature room maintained at 30°C and 60% humidity. Queens were not fed until the first workers eclosed. Thereafter, queens were fed with small amounts of Bhaktar-Whitcomb diet (Bhaktar and Whitcomb 1970) supplemented with amino acids and vitamins, crickets, and mealworms.

Male and female reproductives often linger around the nest entrance during the days preceding the mating flight (Hölldobler 1976, Gordon 1995). For cuticular hydrocarbon analysis, males were collected from their parent colonies before the 2002 mating flight. Each male was collected from a different parent colony, and immediately frozen at −20°C.

Collection of eggs, larvae, and pupae

A total of 22 queens maintained in the laboratory were used in this study. Eggs were collected from all queens; the day these eggs were laid was not known. A portion of these eggs was frozen at −20°C, while the rest was used for DNA extraction immediately upon collection. From each queen, both fresh and frozen eggs were extracted. To obtain a sample of eggs for which the day the egg was laid was known, all eggs were removed from 13 of the 22 queens. During the following week, all newly laid eggs were collected once a day from these 13 queens. A total of 137 eggs that were at most 24 hours old were collected (hereafter referred to as 24-hr eggs; all 24-hr eggs were extracted while fresh). In addition, 38 larvae were collected from 14 of the 22 queens. Finally, one pupa was collected from each of 10 queens.

Microsatellite analysis

Of 413 eggs on which DNA extractions were performed, 150 had been frozen at −20°C, while 263 were extracted while fresh. After testing a number of different DNA extraction procedures, the majority of eggs were extracted by adding 50 μL of 20% Chelex solution (Bio-Rad, Hercules, California, USA) to each egg, and incubating at 95°C for 20 min. The samples were then centrifuged for 2 min, and the supernatant containing the DNA was removed. Larval DNA was extracted using 150 μL of 10% Chelex, otherwise proceeding as with eggs. DNA was extracted from pupae using QIAquick PCR purification columns (Qiagen, Valencia, California, USA). After removing the abdomen, each pupa was crushed using a teflon pestle. Following overnight digestion with proteinase K (0.5 mol/L EDTA, pH 8.0; 0.5% SDS; 100 μg/mL proteinase K), samples were centrifuged for 1 min, and 125 μL of supernatant were purified according to the QIAquick PCR purification kit protocol.

To determine whether DNA had been successfully extracted from the eggs, larvae, and pupae, each extract was amplified at either Pb9 (Volny and Gordon 2002*b*) or *Myrt3* (Evans 1993). PCR products were run on 1.2% agarose gels, stained with ethidium bromide, and visualized under UV light. All successful DNA extractions were amplified once at microsatellite loci *Myrt3* and *Pb8*, using reverse primers which were fluorescently labeled with 6-FAM (Operon, Huntsville, Alabama, USA). PCR conditions were as described in Volny and Gordon (2002*a*, *b*). PCR products were run on 5% SequaGel (National Diagnostics, Atlanta, Georgia, USA) acrylamide gels on an ABI 377 automated sequencer (Applied Biosystems, Foster City, California, USA). Gels were analyzed using GeneScan v. 3.1.2 software (Applied Biosystems).

An individual's genotype at *Myrt3* predicts its reproductive caste (Volny and Gordon 2002*a*). Like *Myrt3*, microsatellite locus *Pb8* is also diagnostic of reproductive caste in this population of *P. barbatus*. Eggs, larvae, and pupae were classified as inter-lineage or same-lineage based on their *Myrt3* and/or *Pb8* genotype. Since different numbers of eggs and larvae were collected from each queen, statistical analyses were performed using the proportion, rather than absolute number, of each queen's eggs and larvae that exhibited inter-lineage or same-lineage genotypes. A Spearman rank correlation was used to test whether the proportion of same-lineage genotypes decreases over each successive developmental stage (24-h eggs, eggs, larvae, pupae). All pairwise comparisons of the proportion of same-lineage genotypes in each developmental stage were performed using one-tailed Mann-Whitney *U* tests.

Cuticular hydrocarbon analysis

To assign males used for the chemical analysis to a lineage, DNA was extracted from heads, and amplified at microsatellite locus *Myrt3*, as described in Volny and Gordon (2002*a*). Males were assigned to lineage J1 or lineage J2 based on their *Myrt3* genotype.

Cuticular hydrocarbons were sampled from decapitated males using solid phase microextraction (SPME; Supleco, Bellfonte, Pennsylvania, USA), a solvent-less method for the extraction of cuticular lipids (Monnin et al. 1998, Sledge et al. 2000). The majority of *P. barbatus* cuticular lipid compounds are hydrocarbon molecules (Wagner et al. 1998). Thawed ants were held with forceps while a SPME fiber was rubbed on the cuticle of the dorsal side of the abdomen for 3 min to avoid

contamination from internal or glandular sources of lipids. Thirteen males were analyzed, four from lineage J1 and nine from lineage J2.

Samples were analyzed using gas chromatography (Varian 3900 with flame ionization detector; Varian, Palo Alto, California, USA). The SPME fiber was inserted into the injector and surface lipids were desorbed onto a capillary column (DB-1 fused silica, 30 m, 0.25 ID, 0.25 μm film thickness; J&W Scientific, Folsom, California, USA) for 5 min at 310°C (samples purged after 90 s; helium carrier gas at 1mL/min flow rate). During injection, the oven temperature was held at 170°C for 5 min and was then raised to 220°C at 25°C/min and then to 310°C at a rate of 3°C /min with a 5-min hold. Elution patterns and retention times from male samples were compared to worker samples for which the cuticular hydrocarbons had been previously identified (Wagner et al. 1998), to confirm that the peaks used in the statistical analysis were in fact cuticular hydrocarbons.

The amount of each hydrocarbon component was calculated as the area under each peak in the total ion chromatogram. Prior to analysis, data for each compound were transformed using the equation $Z_{ij} = \ln(Y_{ij}/g(Y_j))$, where Z_{ij} is the standardized peak area i for individual j, Y_{ij} is the observed peak area i for ant j, and $g(Y_j)$ is the geometric mean of all peak areas used in the analysis for ant j (Aitchison 1986, Dietemann et al. 2003). Linear discriminant analysis (SPSS 2002) was used to determine if ants of different lineages had different relative abundances of cuticular hydrocarbon compounds. Following the methods of Wagner et al. (1998), the relative abundances of the five most abundant compounds were used in the analysis. We limited the number of hydrocarbon peaks used in the analysis to five in order to avoid significant discrimination where none exists; such false discrimination can occur when there are large numbers of independent variables relative to the sample size (Panel on Discriminant Analysis and Clustering 1989). Discriminant scores were calculated for each ant as the position along a new axis that represents the linear combination of variables providing the best discrimination among groups. Overall effectiveness of the discrimination was tested by using a Wilks' lambda test. Discriminant scores were then used to predict group membership.

Results

Reproductive caste of brood

Founding dependent lineage queens laid eggs of same-lineage, reproductive genotype. The mean proportion of same-lineage genotypes among 24-h eggs, averaged over the 13 queens from which 24-hr eggs were collected, was 19% (range 0–50%, standard deviation 0.21). Samples of each developmental stage were categorized as inter-lineage (worker) or same-lineage (reproductive) based on their *Myrt3* and *Pb8* genotypes. Because genotyping of eggs was difficult, we included samples that were genotyped at *Myrt3*, *Pb8*, or both, in the data set. In four of 134 eggs genotyped, *Myrt3* and *Pb8* genotypes disagreed. Because assignment of reproductive caste is inconclusive in such cases, these four eggs were omitted from analyses. None of the 134 eggs were haploid.

The proportion of same-lineage genotypes decreased as the brood developed. The mean proportion of same-lineage genotypes among eggs of unknown age was 15% (range 0–67%, standard deviation 0.24). Among larvae, the mean proportion of same-lineage genotypes was 11% (range 0–100%, SD = 0.29). None of the pupae had same-lineage genotypes. This represents a significant decrease in same-lineage genotypes over successive developmental stages (Spearman rank correlation, $r = -0.39$; $P = 0.0035$).

Of 38 larvae genotyped, five had same-lineage genotypes. Four of the five same-lineage larvae were collected from the same queen. All of this queen's larvae were same-lineage. Curiously, this queen had both same-lineage and inter-lineage genotypes among her eggs. During the 12 months in which this queen was kept in the laboratory, she always had an egg pile with several larvae, yet none of the larvae grew to considerable size, or developed into pupae.

Success of DNA amplification from eggs

While DNA extraction from larvae and pupae was not problematic, the success of DNA extractions from eggs varied depending on the type of egg used (fresh vs. frozen), and the microsatellite locus used to test the extract. Only 24 of 150 PCRs of DNA extracted from frozen eggs yielded a PCR product (16%), while 142 of 263 PCRs of DNA extracted from fresh eggs were successful (55%). Of the fresh eggs extracted using the same extraction procedure (50 μL of 20% Chelex; see *Methods*), 40% of PCRs using primers for locus *Myrt3* were successful, while 73% of PCRs using primers for microsatellite locus *Pb9* (Volny and Gordon 2002*b*) were successful. The reasons for this difference in amplification success are unclear; there was no difference in amplification success of *Myrt3* and *Pb9* for DNA extractions of adult tissue.

Differences in male cuticular hydrocarbons

Males from different genetic lineages differ in their cuticular hydrocarbon profiles. The two genetic lineages could be discriminated by differences in the relative abundances of the five most abundant cuticular hydrocarbon compounds (one discriminant function: canonical correlation = 0.975; Wilks' $\lambda = 0.242$, $F_{5,12} = 12.788$, $P < 0.012$; Fig. 1). Discriminant scores from this analysis misclassified one of 13 males into the other genetic lineage.

Discussion

This study demonstrates that founding dependent lineage queens lay eggs of same-lineage, reproductive genotype. Thus, founding queens do not fertilize their

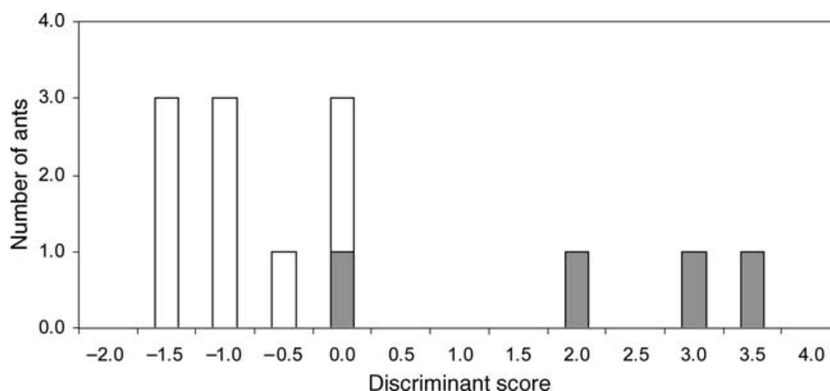

FIG. 1. Frequency distribution of linear discriminant analysis scores of cuticular hydrocarbon profiles for ants from the two genetic lineages. The discriminant scores represent the position of each ant along a new axis, the linear combination of variables that best discriminates between the groups. Males with similar discriminant scores have similar overall hydrocarbon profiles. Open bars represent males of lineage F; gray bars represent males from lineage J2.

eggs exclusively with sperm from the opposite lineage. We can exclude the possibility that unfertilized (haploid) eggs were mistakenly classified as same lineage because of their *Pb8* genotypes. Unlike microsatellite locus *Myrt3*, which has two alleles (one of which is extremely rare) in lineage J2, we have observed ten alleles in lineage J2 at locus *Pb8* (V. P. Volny, *unpublished data*). All eggs and larvae classified as same lineage were heterozygous at *Pb8*, and were thus not haploid. It appears that eggs of same-lineage genotype do not complete development. None of the same-lineage larvae developed into pupae. Our results are similar to those of Helms Cahan et al. (2004), that same-lineage progeny of founding queens have an extremely low probability of successful development.

The inability of same-lineage genotypes to complete development could be due to the lack of an essential nutrient or other environmental factor. Alternatively, it could be due to active removal of same-lineage genotypes by the queen or workers. It has been shown, in other ant species, that workers are able to assess the sex of developing brood (Aron et al. 1994, 1995, Sundström et al. 1996). Workers of the ant *Myrmecia gulosa* can discriminate queens and fertile workers from infertile individuals based on their hydrocarbon profiles (Bonavita-Cougourdan et al. 1991, Dietemann et al. 2003). If dependent lineage queens and/or workers can identify the reproductive caste of developing brood, they could eliminate those destined to become reproductives. In many developing colonies, injured eggs, larvae, and pupae are immediately eaten by mature members of the colony (Wilson 1971). In species with genetic caste determination, same-lineage eggs may be recycled in a similar way.

P. barbatus colonies may not only feed on eggs of reproductive genotypes, but may also benefit from the production of trophic eggs by founding queens. Trophic eggs are produced for consumption, and cannot develop into larvae (Wilson 1971). They are used to store nutrients and redistribute food within a colony (Gobin and Ito 2000), and often form the primary source of nutrition for developing larvae (Hölldobler and Wilson 1990, Tay and Crozier 2000). As in other species of Hymenoptera, *P. barbatus* larvae frequently feed on eggs (V. P. Volny, *personal observation*). In some cases, trophic eggs may lack detectable DNA (Voss 1981). This may explain why at most 73% of egg PCRs were successful, compared to 100% success of PCRs of larvae and pupae.

Mating aggregations of *Pogonomyrmex* harvester ants have been compared to the lek mating systems of many vertebrates: males gather to attract females, and then compete for mates (Hölldobler 1976, Davidson 1982). Many males may engage in intense competition for access to a single queen. It would clearly benefit a queen to be able to identify and mate with males from both lineages, allowing her to produce workers as well as female reproductives (Ashe and Oldroyd 2002). Though mate choice may not be under the queen's control, she does control when she leaves the mating aggregation to found a colony. A queen may be able terminate mating once she has mated with a male of each lineage, or once a favorable ratio of intra- vs. inter-lineage matings has been achieved.

Our chemical analysis of *P. barbatus* males shows that there are measurable differences in the cuticular hydrocarbon profiles of the two genetic lineages. *P. barbatus* workers have been shown to utilize cuticular hydrocarbons to discriminate nestmates from non-nestmates (Wagner et al. 2000), and to recognize the tasks of other ants they encounter (Greene and Gordon 2003). Boomsma et al. (2003) found within-colony patriline differences in hydrocarbon profiles in another ant species. This study shows that dependent lineage queens could distinguish between males from different genetic lineages using the males' cuticular hydrocarbons.

Over the course of her lifetime, a queen produces many times more workers than reproductives (Gordon 1992, Gordon and Wagner 1997, Wagner and Gordon

1999). Preferential mating with males of the opposite lineage could help a queen produce the observed ratio of workers to reproductives. As Hosken and Pitnick (2003) point out, some form of sperm choice (Birkhead 1998) would offer additional advantages, allowing the queen to manage temporal variation in the need to produce female reproductives. Selection for a queen's ability to distinguish between males from different lineages should be strong, even without a capacity for sperm choice (Ashe and Oldroyd 2002).

The results from this study clearly demonstrate post-zygotic culling of reproductives. However, we were surprised to find no more than 19% reproductive genotypes among eggs. In the absence of sperm choice, if mating is random with respect to lineage, males from each lineage are equally frequent, and each male contributes the same amount of sperm, one would expect an equal ratio of inter-lineage to same-lineage genotypes among the eggs. Under this scenario of random mating, a deviation from equal proportions of worker to reproductive eggs would be expected if either queens have some control over which sperm are used to fertilize eggs (Hosken and Pitnick 2003), or some eggs of reproductive genotype were eaten by queens, workers, or fed to developing larvae. An alternative explanation for the observed proportion of reproductive genotypes is that females preferentially mate with males of the opposite genetic lineage. For example, if a queen were to mate with four times as many males of the opposite lineage, relative to males of her own lineage, 20% of her eggs would be reproductive if sperm use is random. Indeed, such overmating with males of the opposite lineage could be adaptive as a queen produces more workers than reproductives over the course of her lifetime. The results presented here do not preclude the possibility of pre-zygotic selection by the queen. If queens can select sperm, the worker to reproductive ratio among newly laid eggs should differ from the ratio of sperm of the two lineages in the queen's spermatheca. It would be informative to analyze the sperm content of the queens' spermathecae in future work.

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